MOLECULAR AND CELLULAR BIOLOGY, Sept. 2001, p. 5767–5777 Vol. 21, No. 17
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.17.5767–5777.2001
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Control of DNA Rereplication via Cdc2 Phosphorylation Sites in the Origin Recognition Complex

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Received 28 March 2001/Accepted 7 May 2001

Cdc2 kinase is a master regulator of cell cycle progression in the fission yeast *Schizosaccharomyces pombe*. Our data indicate that Cdc2 phosphorylates replication factor Orp2, a subunit of the origin recognition complex (ORC). Cdc2 phosphorylation of Orp2 appears to be one of multiple mechanisms by which Cdc2 prevents DNA rereplication in a single cell cycle. Cdc2 phosphorylation of Orp2 is not required for Cdc2 to activate DNA replication initiation. Phosphorylation of Orp2 appears first in S phase and becomes maximal in G_2 and M when Cdc2 kinase activity is required to prevent reinitiation of DNA replication. A mutant lacking Cdc2 phosphorylation sites in Orp2 (*orp2-T4A*) allowed greater rereplication of DNA than congenic *orp2* wild-type strains when the limiting replication initiation factor Cdc18 was deregulated. Thus, Cdc2 phosphorylation of Orp2 may be redundant with regulation of Cdc18 for preventing reinitiation of DNA synthesis. Since Cdc2 phosphorylation sites are present in Orp2 (also known as Orc2) from yeasts to metazoans, we propose that cell cycle-regulated phosphorylation of the ORC provides a safety net to prevent DNA rereplication and resulting genetic instability.

Cyclin-dependent kinases (CDKs) are essential activators of replication initiation and are also required for once-per-cell-cycle control of DNA replication. Eukaryotic DNA replication initiates at many replication origins so that many replication forks can work simultaneously. This allows replication of large genomes in a short S phase. However, replication of each origin must be limited to once per cell cycle to maintain ploidy and genome stability.

In the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, initiation depends on DNA sequences called autonomously replicating sequences. To function as a replication origin, autonomously replicating sequence DNA must bind the origin recognition complex (ORC) (3). The ORC has six protein subunits named Orc1 through Orc6 and is an essential replication initiator in yeasts as well as metazoans (reviewed in reference 64). At least in some systems, the ORC stays bound to DNA throughout the cell cycle (2, 17, 45, 57). Beginning in late M or early G_1 phase, the ORC recruits additional proteins so as to form a large preinitiation complex (reviewed in reference 54). One of the first of these additional proteins is Cdc18 (*S. pombe* name; also known as Cdc6 in *S. cerevisiae*) (11, 42, 63). Cdc18 is likely to be an ATPase, and is thought to function with the ORC to load the six MCM proteins (Mcm2 through Mcm7) onto the chromatin (12, 61). The MCMs may serve as the replicative helicase (reviewed in reference 72). Other essential initiator proteins that associate with the ORC include Cdc45, Mcm10, and Cdt1 (reviewed in references 39 and 70).

The preinitiation complex is apparently fully assembled in G_1 phase, and yet initiation does not occur. Initiation is not triggered until two protein kinases become active. These kinases are Hsk1 (*S. pombe* name; also known as Cdc7) and Cdc2 (also known as Cdc28 in *S. cerevisiae*). Cdc2 is a cyclin-dependent kinase, and at different times of the cell cycle is activated by various different cyclins (reviewed in references 31, 48, and 67).

In *S. pombe,* much of the cell cycle is controlled by Cdc2 and its various cyclin partners. For instance, Cdc2-cyclin complexes control not only DNA replication, but also growth polarity, spindle pole body duplication, chromosome condensation, mitotic spindle functions, mitosis, and cytokinesis (56). Though much has been learned about how Cdc2 kinase itself is regulated, relatively little is understood about how this kinase in turn regulates the many events of the cell cycle, and it is not well-known what proteins are phosphorylated by Cdc2 to accomplish the many cell cycle transitions under Cdc2 control. In particular, while it is clear that initiation of replication depends on Cdc2 kinase activity, it is not known what proteins are phosphorylated by Cdc2 to trigger replication. In view of the global regulation of the cell cycle by Cdc2, regulation of replication by Cdc2 might be indirect. However, experiments in *S. cerevisiae* provide strong evidence that Cdc28 activity is required throughout S phase for the firing of individual replication origins, and this suggests a direct involvement (19).

Cdc2 kinase activity is low during G_1 phase when the preinitiation complex is assembled, in part because of a lack of cyclin and in part because of high levels of the Cdc2 inhibitor Rum1. Later, after assembly of the preinitiation complex, Cdc2 kinase is activated when the cyclins Puc1 and Cig2 are synthesized and when Rum1 is degraded. This Cdc2 kinase activity (along with Hsk1 kinase activity) allows the preinitiation complex to fire, triggering S phase (24, 46, 51). Cyclins accumulate during S phase and G_2 (15), but full Cdc2 activity is held in check by inhibitory phosphorylation by the Wee1 and Mik1 kinases. At the end of G_2, Cdc2 binds the mitotic cyclin Cdc13 and is dephosphorylated by the Cdc25 phosphatase. This generates a high level of Cdc2 kinase activity sufficient for mitosis (reviewed in reference 4). At the end of mitosis, Cdc2 is inactivated by several mechanisms, including destruction of cyclin, expression of Rum1, and possibly removal of the activating T-loop phosphorylation (5, 13, 41, 52).

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Strikingly, the low level of Cdc2 kinase activity in G_1 is probably essential for assembly of the preinitiation complex; that is, when Cdc28 kinase is activated in G_1 by some experimental manipulation, it prevents formation of the preinitiation complex (63). In addition, if Cdc2 or Cdc28 kinase activity is artificially lowered in G_2, then preinitiation complexes re-form and can fire, leading to rereplication (8, 16, 27). Thus, the cycle in Cdc2 kinase activity (low in G_1 phase, moderate in S and G_2, high in M) is tightly linked to the cycle in replication. The low kinase activity in G_1 allows preinitiation complexes to form, and the appearance of Cdc2 kinase activity in S allows them to fire. At the same time, this moderate or high Cdc2 kinase activity in S, G_2, and M prevents preinitiation complexes from re-forming, and so rigorously prevents any rereplication.

In light of the links between replication and Cdc2 kinase, it is notable that several components of the preinitiation complex have conserved clusters of sites for Cdc2 phosphorylation. These components include Orp2 (also known as Orc2), Cdc18 (also known as Cdc6), Mcm4, and Mcm10. At present, there is no evidence that phosphorylation of any of these sites is involved in triggering initiation. However, there is evidence that some of these sites in MCMs and Cdc18 help prevent DNA rereplication by preventing assembly of new preinitiation complexes once S phase has begun. In particular, Cdc18 phosphorylated by Cdc2 is inactivated via ubiquitin-mediated degradation (21, 23, 30, 32, 37). Degradation is not the only way to inactivate Cdc18, and in human and *Xenopus laevis* cells, much of the Cdc6 is not degraded but is exported from the nucleus apparently as a result of CDK phosphorylation (60, 62, 66). In budding yeast, phosphorylation of Cdc6 and MCMs probably masks nuclear localization signals on these proteins, rendering them cytoplasmic (33, 38, 55). Cdc18 inactivation and inhibition of MCM function are two mechanisms for preventing assembly (or reassembly) of the preinitiation complex once Cdc2 kinase is active.

Although the Cdc2 phosphorylation sites in Cdc18 are involved in preventing reinitiation, they certainly are not the only such mechanism, since mutant proteins lacking the phosphorylation sites do not allow rereplication (at least when expressed at wild-type levels) (21, 44, 60, 71). Furthermore in *S. pombe*, unlike *S. cerevisiae*, the MCM proteins are constitutively nuclear (28, 55, 58, 59, 68). Thus, there must be additional, undiscovered controls preventing reinitiation. One such control in higher eukaryotes involves binding of an inhibitor, geminin, to the Cdt1 factor; however, no analogous pathway is known in yeasts (50, 69, 73). Existence of multiple pathways is not surprising, since even a small degree of reinitiation could have serious effects on genome stability.

We have found that *S. pombe* Cdc2 interacts with the *S. pombe* ORC protein Orp2 (the homolog of Orc2). Based on this observation, we proposed that Cdc2 regulates DNA replication directly at replication origins (40). Orp2 and its homologs have a conserved N-terminal cluster of consensus Cdc2 phosphorylation sites. Thus, since (i) Orp2 binds to Cdc2, (ii) Orp2 has appropriate sites for phosphorylation by Cdc2, and (iii) Cdc2 is known to trigger replication and to prevent rereplication, we asked whether phosphorylation of Orp2 by Cdc2 was involved in these processes. We found that Orp2 is phosphorylated by Cdc2, assessed the phosphorylation of Orp2 through the cell cycle, mutated the Cdc2 phosphorylation sites in Orp2, and investigated the biological impact of these mutations.

MATERIALS AND METHODS

Yeast methods, strains, and plasmids. *S. pombe* methods were essentially as described by Moreno et al. (53): YES is rich medium, and EMM2 is defined medium and was supplemented with leucine (L), uracil (U), adenine (A), and histidine (H) as indicated. YSO is rich medium low in adenine (36). The vitamin B_1 thiamine (B1) was added to 2.7 mg/liter as indicated. Genotypes are listed in Table 1.

Potential phosphorylation sites [(S/T)P] encoded by *orp2* were mutated by site-directed mutagenesis using PCR. To generate *orp2-T4A*, plasmid pJL207 (*orp2*$^+$ cloned into pREP3 [40]) was used as a template. Primers P1 and P2 amplified the 5′ coding region of *orp2*$^+$; P2 has nucleotide changes that altered Thr to Ala. Separately, primers P3 and P4 were used to amplify the region of *orp2*$^+$ 3′ of the sequence amplified by P1-P2. The P3 primer has nucleotide changes to alter two Thr codons to Ala codons and introduces an *Xho*I site. The P1-P2 and P3-P4 products were gel isolated, phosphorylated, and ligated together, and the ligation product was amplified using P1 and P4. This product was cloned to replace the corresponding region of the *orp2*$^+$, and the clone was sequenced. Resulting amino acid changes are shown in Fig. 1. The mutated region was subcloned into appropriate *orp2*$^+$ vectors as follows: pJL211 (pRep1-GST-orp2 [40]) was changed to make pJL329 (pRep1-GST-orp2-T4A). pJL320 (genomic *orp2*$^+$, *leu1*$^+$ integrating vector [see below]) was changed to make pJL322 (genomic *orp2*-T4A, *leu1*$^+$ integrating vector). Primer sequences were P1 (CTGAAGATACGTTTTCAACCATTTTTAAAG), P2 (CGCCTAAAAAAG GAGTTCTTGAAGAT), P3 (CAACAACTTCTGGTGCTAATGTAGAAGG), and P4 (CTCCTCGAGCACCAGGACATCGAA).

To express alleles of *orp2* from the *orp2* promoter in the genome, an *orp2*$^+$ *leu1*$^+$ vector (pJL320) was constructed. pJL320 contains the *orp2* promoter and coding sequence followed by the *nmt1* terminator cloned between the *Pst*I-*Sac*I sites of integrating vector pJK148 (*leu1*$^+$*)* (35). Steps in construction of pJL320 were as follows: The 1.7-kb *Pst*I-*Nde*I fragment from pER17 (40) was cloned into *Pst*I/*Nde*I-digested pJL207 (pREP3-*orp2*$^+$) (40), thus replacing the *nmt1* promoter with the *orp2* promoter. From this, the *Pst*I-*Sac*I fragment containing the *orp2* promoter and coding sequence and the *nmt1* terminator was moved into *Pst*I-*Sac*I-digested pJK148 to create pJL320. pJL320 was cut with *Pfl*MI (in the *orp2* 5′ noncoding region) to target integration at *orp2*Δ*::ura4*$^+$ to create *orp2*Δ*::ura4*$^+$*::orp2*$^+$ (*leu1*$^+$).

Construction of JLP230 *orp2*$^+$ and JLP238 *orp2*-T4A strains was as follows: plasmids pJL320 (*orp2*$^+$ *leu1*$^+$) and pJL322 (*orp2*-T4A *leu1*$^+$) were integrated at the *orp2*Δ*::ura4*$^+$ locus in diploid JLP46. Transformants were sporulated, and strains JLP230 and JLP238 were isolated and checked by Southern blot analysis. JLP306 and 308 are Ura$^-$ derivatives of JLP230 and JLP238 made by one-step gene replacement of the *ura4*$^+$ within the *orp2*Δ*::ura4*$^+$ by transformation with *Hin*dIII-digested pJL378 (pJL378 is an unmarked deletion of *orp2* coding sequences cloned into the *Hin*dIII site of pBluescript). Ura$^-$ transformants were obtained using YES–0.1% 5-fluoroorotic acid to create *orp2*Δ*::ura4*$^+$*::orp2*$^+$ (*leu1*$^+$).

Construction of *nmt1*GST-Cdc18* strains was as follows: *pAL27-GST-cdc18-T4A* has Thr codons at positions 26, 98, 104 and 134 mutated to Ala (44). *pAL27-GST-cdc18-T4A* was linearized with *Stu*I and integrated at *ura4-294* in strain FWP162 *h*$^+$ *leu1-32 ura4-294.* GST-Cdc18-T4A is referred to as Cdc18*. The transformant JLP515 *h*$^+$ *leu1-32 ura4-294::GST-cdc18-T4A*, *ura4*$^+$was selected on plates lacking Ura, and stable integrants were identified as isolates that failed to grow on 0.1% 5-fluoroorotic acid–YES plates. JLP515 was mated to JLP306 (*orp2*Δ*::orp2-T4A leu1*$^+$) to obtain strains JLP518, JLP519, and additional congenic isolates. JLP515 was mated to JLP308 (*orp2*Δ*::orp2*$^+$ *leu1*$^+$) to obtain JLP523, JLP524, and additional congenic isolates. All isolates were obtained by tetrad dissection.

Cdc18 induction and flow cytometry. For induction of Cdc18* from the *nmt1* promoter (49), cells were grown overnight in YES-thiamine. This preculture was used to inoculate EMM2-LAUH-B1 to an optical density at 600 nm (OD_{600}) of 0.1 and grown for at least 10 h at 32°C to an OD_{600} of not more than 1.0. Cells were harvested and washed twice in water to remove thiamine. Cells were reinoculated into EMM2-LAUH (without thiamine) to a calculated OD_{600} of

TABLE 1. *S. pombe* strains used in this study

Strain	Genotype	Source or reference
FWP162	*h*$^+$ *leu1-32 ura4-294*	C. Hoffman
JLP46	*h*$^+$/*h*$^-$ *ade6-M216/ade6-M210 ura4-D18/ura4-D18 leu1-32/leu1-32 his7-366/his7-366 orp2*$^+$/*orp2*Δ::*ura4*$^+$	40
JLP52	*h*$^+$/*h*$^-$ *ade6-M216/ade6-M210 ura4*$^+$/*ura4-D18 leu1-32/leu1-32 his7-366/his7-366 orp2*$^+$/*orp*$^+$	40
JLP166	*h*$^-$ *ade6-M216 ura4-D18 leu1-32 his7-366 orp2*Δ::*ura4*$^+$ *porp2TKade*	36
JLP230	*h*$^-$ *ade6-M216 ura4-D18 leu1-32 his7-366 orp2*Δ::*ura4*$^+$::*orp2*$^+$ (*leu1*$^+$)	This study
JLP238	*h*$^-$ *ade6-M210 ura4-D18 leu1-32 his7-366 orp2*Δ::*ura4*$^+$::*orp2-T4A* (*leu1*$^+$)	This study
JLP306	*h*$^-$ *ade6-M216 leu1-32 his7-366 orp2*Δ::*orp2-T4A* (*leu1*$^+$)	This study
JLP308	*h*$^-$ *ade6-M210 leu1-32 his7-366 orp2*Δ::*orp2*$^+$ (*leu1*$^+$)	This study
JLP515	*h*$^+$ *leu1-32 ura4-294*::*GST-cdc18-T4A* (*ura4*$^+$)	This study
JLP518	*ade6-M216 leu1-32 orp2*Δ::*orp2-T4A* (*leu1*$^+$) *ura4-294*::*GST-cdc18-T4A* (*ura4*$^+$)	This study
JLP519	*ade6-M216 leu1-32 his7-366 orp2*Δ::*orp2-T4A* (*leu1*$^+$) *ura4-294*::*GST-cdc18-T4A* (*ura4*$^+$)	This study
JLP523	*ade6-M210 leu1-32 orp2*Δ::*orp2*$^+$ (*leu1*$^+$) *ura4-294*::*GST-cdc18-T4A* (*ura4*$^+$)	This study
JLP524	*ade6-M210 leu1-32 his7-366 orp2*Δ::*orp2*$^+$ (*leu1*$^+$) *ura4-294*::*GST-cdc18-T4A* (*ura4*$^+$)	This study

0.01. These cultures were grown at 32°C and harvested for flow cytometry, Western blotting, and microscopy at the times indicated. Cells were processed for flow cytometry as described (14) except that cells were stained with 1 μM Sytox Green (Molecular Probes) instead of propidium iodide. Fluorescence was analyzed using a Becton Dickinson FACScan and CellQuest software.

Competitive transformations. JLP306 and JLP308 were grown separately overnight at 30°C in 5 ml of YES and then were mixed 1:1, spread on a YES plate, and grown overnight at 30°C. For each transformation, approximately 5 μl of cells from the plate was mixed with 150 μl of 0.1 M lithium acetate (pH 4.7) and incubated at 25°C for 1 h; 0.5 to 1 μg of plasmid DNA was added; 350 μl of 50% polyethylene glycol was added; and cells were incubated overnight at 25°C, harvested, washed gently with EMM2, and resuspended in 1 ml of EMM2. A 5- to 10-μl aliquot of this suspension was diluted and spread for single colonies on EMM2-LAUH (total viable), and the remainder was spread on four to six plates of EMM2-LAH (transformants). Colonies were picked from EMM2-LAH (transformants) or EMM2-LAUH (total) onto YSO. Red or pink colonies on YSO were counted after 2 days.

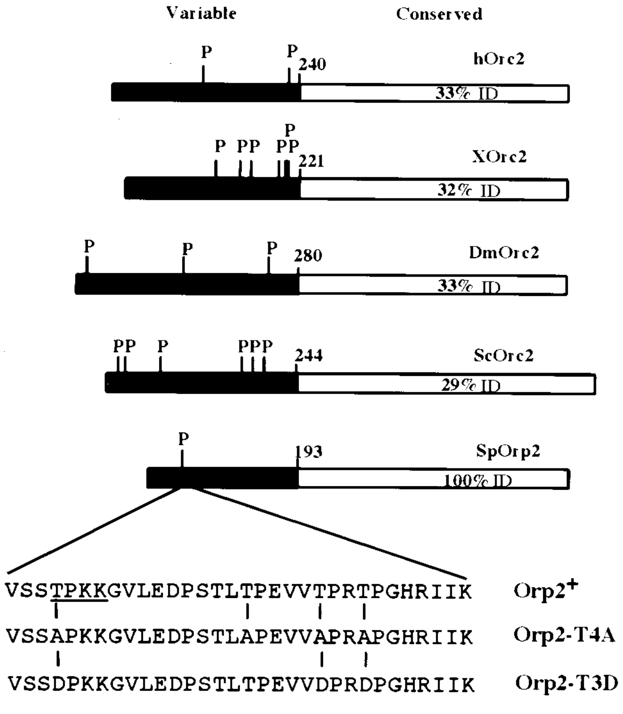


FIG. 1. Cdc2 phosphorylation sites in Orc2 homologs. The N-terminal region shown in black is least conserved. The beginning of the conserved region is indicated (amino acid 193 in Orp2). Bars are drawn to scale. Percent amino acid identity (ID) of the various C-terminal domains compared with Orp2 is shown. P represents consensus Cdc2 phosphorylation sites [(S/T)PX(K/R)]; SP and TP sites that are not perfect matches to the consensus are not shown. The amino acid sequences of Orp2 wild type, Orp2-T4A, and Orp2-T3D show the mutations made, and the single consensus Cdc2 phosphorylation site in Orp2 is underlined. Abbreviations for homologs: h, human; X, *Xenopus*; Dm, *D. melanogaster*; Sc, *S. cerevisiae*; Sp, *S. pombe*.

Antibodies and immunoblotting. GST-Orp2 made in *Escherichia coli* was used to raise rabbit antibodies. The 1.3-kb *Xho*I fragment from pACT-166 (which contains *orp2* cDNA cloned into the two-hybrid vector pAS1) (40) was subcloned into the *Sal*I site of pGEX-KG (Novagen). The resulting glutathione *S*-transferase (GST)–Orp2 fusion protein was produced in *E. coli* (BL21), partially purified by binding to GSH-Sepharose (Pharmacia), and used to raise rabbit polyclonal antisera (26). Crude antisera readily detected Orp2 overexpressed in fission yeast. To detect wild-type levels of Orp2, antibody was affinity purified by binding to purified GST-Orp2 protein immobilized on a nylon membrane. Bound antibodies were eluted with 100 mM glycine (pH 2.0), neutralized with Tris (pH 8.5) and stored in 50% glycerol at −20°C, and used at a 1:100 dilution.

To distinguish which immunoreactive bands were due to Orp2 protein, extract from cells lacking the *orp2*$^+$ gene were compared to extracts from wild-type cells and to extracts from cells overexpressing *orp2*$^+$ (Fig. 2B and data not shown). Because *orp2*$^+$ is essential, selective spore germination was used to obtain the population of cells lacking the gene (*orp2*Δ::*ura4*$^+$), as described (40). Other experiments with epitope-tagged Orp2 have since confirmed the assignment of Orp2 specific bands. Strong cross-reacting bands were not detected when the primary antibody incubation included sarcosyl and sodium dodecyl sulfate (SDS) (Fig. 3A); however, antibody activity was rapidly lost in this solution. Standard Tris-buffered saline–0.3% Tween 20–5% powdered milk (26) was used in all other experiments. For analysis of GST-Cdc18*, whole-cell extracts were made by vortexing with glass beads as described (40), total protein was quantitated using Bradford's reagent (Bio-Rad), and 10 μg of protein was loaded per lane on an SDS–10% polyacrylamide gel. GST-Cdc18* was detected using rabbit anti-GST (gift of L. Hengst) at a 1:500 dilution.

Kinase assays and analysis. GST, GST-Orp2, and GST-Orp2-T4A were expressed in *S. pombe* from plasmids pJL205, pJL211 (40), and pJL329 (this study). GST proteins were isolated from yeast lysates by binding GSH-Sepharose (40), except that lysis and binding were in NETN.5 (50 mM Tris [pH 8], 500 mM NaCl, 5 mM EDTA, 10% glycerol, 1% NP-40) with protease and phosphatase inhibitors as previously described. Bound complexes were washed four times with NETN.5, and GST proteins were eluted with 40 mM glutathione (pH 8.0) and dialyzed against a solution containing 50 mM Tris (pH 8), 150 mM NaCl, 50% glycerol, and 1% NP-40.

Cdc2 kinase was obtained from *S. pombe nuc2* mutant cells arrested at 36.5°C for 4 h. Anti-Cdc13 (gift of P. Russell) bound to protein A-Sepharose or p13^{suc1} beads (R. Aligue and P. Russell) were used to precipitate Cdc2 kinase. Assays were as described (52).

Phosphorylation of GST fusion proteins was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and the ^{32}P-labeled GST-Orp2 and GST-Orp2-T4A bands were excised after autoradiography. Tryptic peptide mapping was performed as described (7) using pH 1.9 buffer for the first dimension of electrophoresis and phosphochromatography buffer for the second dimension of ascending chromatography. Equal amounts of radioactivity were analyzed for each map, so it was necessary to use 1/10 as much of the Cdc2-GST-Orp2 sample as

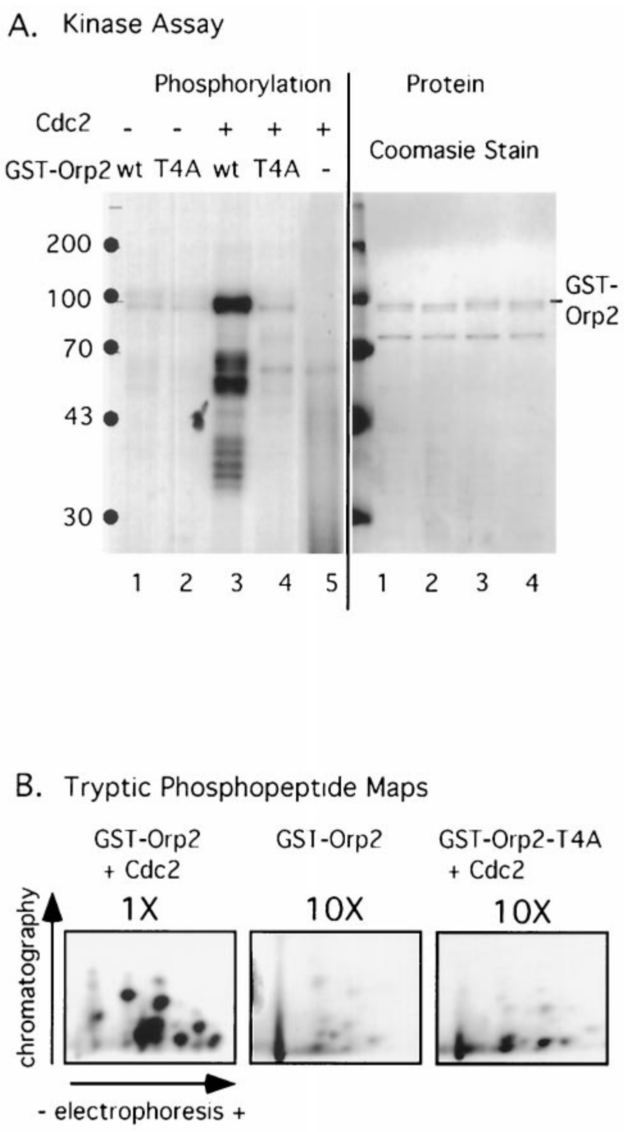


FIG. 2. In vitro phosphorylation of Orp2 by Cdc2 kinase. (A) GST-Orp2$^+$ (wt) and GST-Orp2-T4A (T4A) were incubated with *S. pombe* Cdc2 and [γ-^{32}P]ATP and analyzed by SDS-PAGE. Incorporation of ^{32}P into GST-Orp2 was visualized by autoradiography (left). Purity and relative amounts of GST-Orp2 and GST-Orp2-T4A added to the reactions were seen by Coomassie staining (right). (B) Phosphopeptide maps of proteins from kinase reactions analyzed in panel A. The GST-Orp2 or GST-Orp2-T4A bands were cut out of the gel, digested with trypsin, and analyzed. To visualize phosphopeptides, 10-fold more material (10×) was analyzed for the low-level reactions with GST-Orp2 (no kinase) and GST-Orp2-T4A (site mutant) than for GST-Orp2 plus Cdc2.

for the other samples. Phosphoamino acid analysis was performed as described (7), and results were quantitated using a phosphoimager (Molecular Dynamics).

RESULTS

Cdc2 phosphorylation of Orc2. Consensus Cdc2 phosphorylation sites [(S/T)PX(K/R)] (29) are present in the Orc2 and Orp2 homolog proteins in fission yeast, budding yeast, *Drosophila melanogaster*, *Xenopus*, and mammals (Fig. 1). These sites occur exclusively within the N-terminal regions of the proteins. This region is poorly if at all conserved. Multiple sequence alignments and PSI-BLAST (1) were used to determine an N-terminal boundary for sequence similarity. The first Orc2 sequence motif conserved from yeast to humans, (F/V)(D/E)EYF, begins at amino acid 193 of *S. pombe* Orp2. This conserved motif is the boundary between the variable and conserved regions shown in Fig. 1. Even among mammals, there is a sharp change in sequence conservation between the N-terminal region (64% identical between human and mouse) and the C-terminal region (89% identical between human and mouse). The consensus Cdc2 phosphorylation sites occur exclusively within the variable N-terminal region.

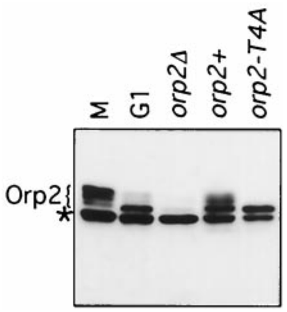


FIG. 3. In vivo modification of Orp2 requires sites needed for Orp2 phosphorylation in vitro. Endogenous Orp2 protein in whole-cell extract was analyzed by SDS-PAGE and immunoblotting using Orp2 antibodies. Several mobility forms of Orp2 were observed. Orp2-specific bands are indicated. A cross-reacting band that is not Orp2 is present in all cells including those lacking Orp2 (orp2Δ) and is marked (*). Lanes are as follows: M, *nuc2* mutant cells arrested in mitosis; G1, *cdc10* mutant cells arrested in G$_1$; *orp2*Δ, selective spore germination and growth of cells lacking *orp2*; *orp2*$^+$, wild type; *orp2-T4A*, mutant in which *orp2-T4A* is the only *orp2* gene in the cells, strain JLP238.

The fact that consensus (and usually additional nonconsensus SP or TP) Cdc2 phosphorylation sites are always present in the N-terminal region of Orc2 homologs, coupled with the fact that Cdc2 kinase is important for regulating DNA synthesis, suggests that these Cdc2 sites could be targets of Cdc2 kinase. The first test of this idea is to find out whether Orc2 is a substrate of Cdc2 in vitro. Therefore, a GST-Orp2 fusion protein was mixed with active Cdc2 kinase from fission yeast, and it was found that the GST-Orp2 became phosphorylated (Fig. 2A). GST-Orp2 was a better substrate than was histone H1, while GST did not become phosphorylated at all (data not shown). Identical results were obtained using Cdc2 isolated from fission yeast lysates by binding to p13^{suc1} beads or by immunoprecipitation of the cyclin Cdc13. These results show that Cdc2 and Cdc13 complexes can phosphorylate Orp2 in vitro.

Phosphorylation of Orp2 in vitro depends on the consensus Cdc2 phosphorylation sites. To see if the phosphorylation of Orp2 in vitro depends on the Cdc2 phosphorylation sites, various (S/T)P sequences in Orp2 were mutated to nonphosphorylatable AP sequences by site-directed mutagenesis. Changing the four TP sequences between amino acids 43 and 66 (Fig. 1) yielded the mutant *orp2-T4A*, which is the mutant characterized in this study. GST-Orp2 and the mutant GST-Orp2-T4A were purified from fission yeast and compared as Cdc2 substrates in vitro. GST-Orp2 was readily phosphorylated, whereas

GST-Orp2-T4A was not (Fig. 2A). Quantitation showed that 10-fold more radioactive phosphate was incorporated into GST-Orp2 than into GST-Orp2-T4A. That equal amounts of substrate proteins were added to each reaction was determined by Coomassie staining of the total proteins in the reactions after separation by SDS-PAGE (Fig. 2A). Hereafter, we will refer to Orp2-T4A as the "nonphosphorylatable" Orp2, even though it might be a substrate of other kinases and could even have weak residual or alternative sites for Cdc2 phosphorylation.

Phosphopeptide mapping showed that the major Cdc2 phosphorylation sites in Orp2 were eliminated by the mutation of four threonines in Orp2-T4A (Fig. 2B). Consistent with the idea that threonines are the phosphorylation targets of Cdc2 in Orp2, phosphothreonine was the main (>90%) phosphoamino acid detected in GST-Orp2 phosphorylated by Cdc2 (datum not shown). Some phosphoserine was also detected in this reaction. However, most of this phosphoserine was independent of the addition of Cdc2 and came from a low level of kinase associated with the GST-Orp2 (datum not shown). This other kinase is not Cdc2, because the phosphopeptides differ from those generated by Cdc2 (Fig. 2B), because no Cdc2 was detected by Western blotting in preparations of GST-Orp2 (data not shown), and because this other kinase phosphorylates GST-Orp2 and GST-Orp2-T4A equally well (Fig. 2A).

Phosphorylation of Orp2 in vivo requires the same sites that are phosphorylated by Cdc2 in vitro. Phosphorylation of Orp2 in vivo causes a shift to a slower-migrating species in SDS-PAGE analysis (45). If the slowly migrating Orp2 results from phosphorylation by Cdc2, then the same Cdc2 sites required for Orp2 phosphorylation in vitro should also be required for phosphorylation and mobility shift of Orp2 in vivo. To test this, isogenic strains were constructed having either wild-type *orp2*$^+$ or *orp2-T4A*. The *orp2-T4A* strains were viable with no obvious phenotype (see below). Immunoblot analysis showed that asynchronous wild-type cells had several mobility forms of Orp2, whereas the mutant *orp2-T4A* had a single, rapidly migrating band (Fig. 3). This single form of Orp2-T4A comigrated with the single, rapidly migrating form of wild-type Orp2 observed in G_1 cells (Fig. 3). Mutation of the single consensus Cdc2 phosphorylation site was not sufficient to abolish the cell cycle-regulated phosphorylation (data not shown). That the same Cdc2 sites required for Orp2 phosphorylation in vitro are also required for generation of slowly migrating forms of Orp2 in vivo suggests that Orp2 is an in vivo substrate of Cdc2.

Cell cycle regulation of Orp2 phosphorylation. To find out when in the cell cycle Orp2 is phosphorylated, we synchronized cells in G_2 using block and release of a *cdc25-22* mutant and followed Orp2 protein through two cell cycles (Fig. 4A). Orp2 shifted from slowly migrating forms at the G_2 block to faster migrating forms at the end of mitosis. The fastest migrating (unphosphorylated) form of Orp2 appeared simultaneously with the peak in septation. Typically, fission yeast cells are in the S phase of the next cell cycle by the end of septation; thus, the peak in septation and in abundance of fast-migrating Orp2 occurs near the G_1/S boundary.

As a second method for examining cell cycle regulation of phosphorylation of Orp2, cells were blocked in various stages of the cell cycle using cell cycle mutants or the replication inhibitor hydroxyurea (HU). Orp2 isolated from the arrested cells was analyzed by Western blotting (Fig. 4B). Orp2 was in the fastest migrating form at the *cdc10* arrest (G_1 phase). More slowly migrating (phosphorylated) forms first appeared in the S phase (*cdc22* and HU arrests) and when the replication factor Cdc18 was inactivated (*cdc18*). In G_2 and early M (*cdc25* and *nuc2*), Orp2 accumulated in the slowest-migrating forms. It is notable that Orp2 is fully modified at the *cdc25* mutant arrest (G_2). Cdc25 is the tyrosine phosphatase required for activating Cdc2 to the high levels required for mitosis. If Cdc2 is the kinase modifying Orp2, then the pool of Cdc2 that phosphorylates Orp2 may not require activation by Cdc25.

The phosphorylation state of Orp2 was assayed in *cdc2* mutant cells. Phosphorylation of Orp2 was reduced but not abolished in *cdc2-L7* and *cdc2-33* mutants at restrictive temperature (Fig. 4B). Because Cdc2 is essential both for G_1-to-S and G_2-to-M transitions, these *cdc2* mutants arrest as a mixture of 10 to 20% G_1 cells and 80 to 90% G_2 cells. These data show that inactivation of Cdc2 that is sufficient to arrest cell cycle progression does not result in fully dephosphorylated Orp2.

The phosphorylation state of Orp2 was also assayed in cells lacking *cdc13*. Cdc13 is a B-type cyclin essential for mitosis and for once-per-cell-cycle control of DNA replication. Selective spore germination was used to obtain a culture of cells lacking Cdc13. These cells cannot proceed past G_2 into mitosis, and they rereplicate DNA. The Orp2 in these cells was in the fast-migrating (unphosphorylated) form (Fig. 4C). Since cells arrested in G_2 by other mutations (Fig. 4B) accumulate phosphorylated forms of Orp2, the lack of Orp2 phosphorylation in the *cdc13* mutant suggests that the Cdc13-Cdc2 complex may be directly required for phosphorylation of Orp2. Furthermore, this observation suggests that phosphorylation of Orp2 may not be needed for replication, since *cdc13* deletion mutants replicate and rereplicate DNA (27).

Selective spore germination was also used to examine the role of another kinase, Hsk1, in generating phosphorylated Orp2. Hsk1 is essential for DNA replication and is the fission yeast homolog of Cdc7 (47). Cdc7 is bound to replication origins via the ORC and is required for replication initiation at individual origins throughout the S phase (6, 18, 20, 25). Orp2 is fully shifted to the phosphorylated forms even in the *hsk1* mutant (Fig. 4C). Furthermore, Hsk1 failed to phosphorylate Orp2 in vitro (data not shown). These results indicate that Hsk1 does not phosphorylate Orp2. This is consistent with the idea that the relevant Hsk1 substrate is Mcm2 and possibly additional MCMs (reviewed in reference 70).

Phosphorylation of Orp2 is not required for DNA replication. Cdc2 is essential for initiation of replication, and so presumably it has some substrate that is important for initiation. We and others expected that Orp2 could be such a substrate (40, 45). Our data suggest otherwise. Cells carrying *orp2-T4A* as their only allele of *orp2* were viable and healthy, with no obvious phenotype. Likewise, cells with *orp2-T4A* and also mutated at two additional (S/T)P sites (T159→A and S162→A) were also viable and healthy. Finally, cells expressing *GST-orp2-Δ1-127* as the only allele of *orp2* were viable and healthy. This GST-Orp2-Δ1-127 fusion protein lacks the first 127 amino acids of Orp2 (approximately two-thirds of the variable region), including all the T-P sequences mutated in *orp2-T4A*. Except for S139, which has not been tested, all (S/T)P sequences in the variable region of Orp2 can be mu-

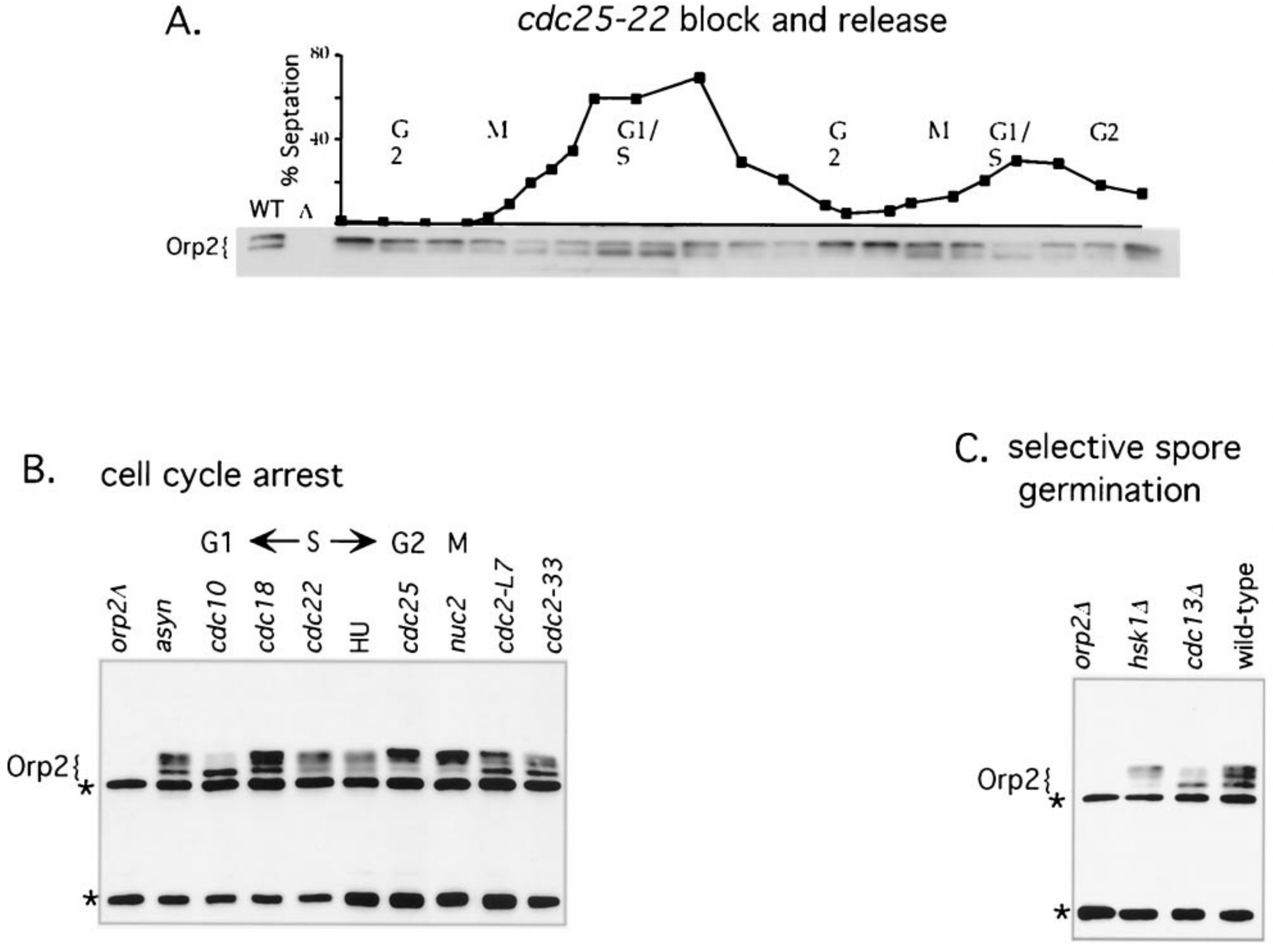


FIG. 4. Cell cycle-regulated phosphorylation of Orp2. (A) Orp2 protein throughout the cell cycle. Cells were synchronized by block and release of a *cdc25-22* mutant. Samples were harvested for analysis every 20 min through two consecutive cell cycles. Whole-cell extracts were analyzed by SDS-PAGE and immunoblotting using affinity-purified Orp2 antibodies. Synchrony and cell cycle progression were determined by septation index (above). Asynchronous cells (WT) and an *orp2* deletion control (Δ) on the left show which bands are Orp2 specific; the *orp2*Δ was made by selective spore germination (see Materials and Methods). (B) Orp2 protein during cell cycle arrest. Mutants were arrested by shift to 36.5°C for 4 h, the mutant is indicated above each lane, and the stage of cell cycle arrest is shown at the top. Orp2 bands are indicated. Cross-reacting bands that are not Orp2 are marked (*). Abbreviations: asyn, asynchronous wild type shifted to 36.5°C for 4 h; HU, wild type incubated with 12 mM HU for 4 h at 32°C; *orp2*Δ, cells with *orp2* deletions from selective spore germination of an *orp2*Δ/*orp2*$^+$ heterozygote lack Orp2-specific bands. (C) Orp2 protein in cells lacking essential genes. Immunoblot analysis of Orp2 from cells obtained by selective germination of spores with deletions of essential genes *orp2*, *cdc13*, and *hsk1* as indicated. wild-type, selective spore germination of a wild-type diploid heterozygous at *ura4* (*ura4-D18*/*ura4*$^+$, JLP52).

tated or deleted without significantly altering replication initiation.

The *orp2-T4A* allele was integrated into the chromosome at the *orp2*Δ*::ura4*$^+$ locus under control of the *orp2* promoter. Several experiments were done to see if this mutant had a defect in initiation. Flow cytometry showed that the S phase progressed similarly in *orp2-T4A* and wild-type cells (data not shown). There was no detectable change in cell size at division, which would have indicated a cell cycle delay. There was no genetic interaction between *orp2-T4A* and *cdc18, cdc19,* or *cdc21* temperature-sensitive alleles defective for replication factors.

To perform a possibly more sensitive assay, we compared the efficiency with which *orp2-T4A* and wild-type strains could be transformed with an *ars* plasmid as has been done to evaluate *ars* sequences in *S. pombe* (10, 22). The assays described above may be insensitive detectors of initiation defects, because each chromosome has many origins. Even if the efficiency of initiation declined to, say, 30% of wild-type values, this 30% may be adequate to give a wild-type phenotype by growth rate or flow cytometry assays, etc. Since plasmids have only a single origin, any defect in origin usage lowers transformation frequency. To control for other factors affecting transformation and cell viability, the mutant and wild-type strains were mixed and transformed in one tube (Materials and Methods). The *orp2-T4A* strain was marked with *ade6-M210*, and the *orp2*$^+$ strain was marked with *ade6-M216*. Both alleles are Ade$^-$, but on rich medium low in adenine (YSO), *ade6-M216* yields pink colonies while *ade6M-210* yields red colonies. The *orp2*$^+$ *ade6-M216* and *orp2-T4A ade6-M210* strains were mixed 1:1, grown over-

TABLE 2. Transformation efficiencies of *orp2-T4A* and *orp2-T3D* strains relative to *orp2*$^+$

Strains compared	Plasmid[a]	Relative *ars* function[b]	Ratio of[c]:		Relative transformation[d]
			Colonies	Transformants	
orp2-T4A/*orp2*$^+$	*ars*3002	100	125/176	383/189	2.9
	*ars*3002Δ5	40–50	131/139	388/144	2.9
	*ars*3002Δ9	20–30	201/227	266/180	1.6
	*ars*3002Δ11	10–20	302/277	303/266	1.4
	*ars*3002Δ8	<5	134/149	283/138	1.9
orp2-T3D/*orp2*$^+$	*ars*3002	100	92/91	80/120	0.7
	*ars*3002Δ5	40–50	84/103	79/120	0.8
	*ars*3002Δ9	20–30	190/223	93/107	1.0
	*ars*3002Δ11	10–20	92/115	93/107	1.1
	*ars*3002Δ8	<5	75/119	76/124	1.0

[a] Plasmids are described in reference 22.

[b] *ars*Δ/*ars*3002 × 100 (data from reference 22).

[c] Number of red (*orp2* mutant)/pink (*orp2*$^+$) patches on YSO from colonies picked from nonselective medium (Colonies) or medium lacking uracil (Transformants).

[d] Ratio of transformants/ratio of colonies.

night, and then transformed with *ura4*$^+$ tester plasmids containing wild-type and mutant derivatives of *ars3002* (Table 2) (22). Transformants were selected on medium lacking uracil. In addition, cells were plated on medium containing uracil to determine the number of cells of each genotype surviving the transformation protocol. Colonies from both surviving cells and from transformed (i.e., Ura$^+$) cells were picked to YSO to determine whether they were *orp2*$^+$ *ade6-M216* or *orp2-T4A ade6-M210*. The ratio of transformants of each genotype was divided by the ratio of surviving cells to normalize for survival and determine the relative transformation efficiency (Materials and Methods).

As shown in Table 2, the *orp2-T4A* mutant had transformation efficiencies up to threefold higher than the wild-type strain. The plasmids used in this assay contained wild-type and mutant derivatives of *ars3002.* This test was performed with several different *ars* constructs. Deletion mutants of *ars3002* were chosen because they have been extensively characterized (22). In fact, the wild-type *ars* showed the greatest difference between *orp2-T4A* and *orp2*$^+$. Perhaps the differences detected in this assay are obscured by the defective function of the *ars* mutants. In any case, the results give no indication of any initiation defect in the mutant, but rather suggest that the Cdc2 phosphorylation sites in Orp2 could have an inhibitory function.

Introducing mutations that might mimic phosphorylation of Orp2 had little effect on Orp2 function. Threonines in three of the putative Cdc2 phosphorylation sites were changed to aspartate (Fig. 1). The resulting mutant, *orp2-T3D,* has no obvious defect in growth or replication (data not shown). This *orp2-T3D* mutant has a transformation efficiency 0.7- to 1.1-fold that of the wild type in the transformation assay (Table 2); the variations in relative efficiency depended upon the plasmid *ars* tested. As with *orp2-T4A*, the greatest difference in transformation efficiency was observed with the wild-type *ars*, although in this case, the difference is a decrease in transformation efficiency in the mutant; however, all experiments suggest the *orp2-T3D* mutant is wild type or nearly wild type for initiation.

The Cdc2 phosphorylation sites in Orp2 help prevent rereplication. Cdc2 is essential for limiting DNA replication to once per cell cycle. Inactivation of Cdc18 is one mechanism used by Cdc2 to prevent reinitiation, but there must be redundant mechanisms, since loss of the phosphorylation sites on Cdc18 does not in itself allow reinitiation. In *S. pombe*, overexpression of Cdc18 does allow rereplication, probably by supplying functional Cdc18 and also by interfering with other pathways for control of rereplication. Phosphorylation of Orp2 might be one of these additional mechanisms. If so, then we expect no obvious phenotype of mutating Cdc2 phosphorylation sites in Orp2 in wild-type cells, but the role of these sites in regulating rereplication might be revealed if Cdc18 is de-regulated. Therefore, we compared rereplication in *orp2-T4A* and wild-type strains when *cdc18** was overexpressed in each of them. *cdc18** lacks four Cdc2 sites and induces reinitiation and rereplication when overexpressed (as does *cdc18*$^+$ itself) (44).

The *orp2-T4A* mutation enhanced rereplication induced by overexpression of *cdc18**. A strain with a stable integrated copy of *cdc18** controlled by the inducible *nmt* promoter was crossed with isogenic *orp2-T4A* and wild-type *orp2*$^+$ strains that have wild-type levels of Orp2 expressed from its endogenous promoter. *nmt-cdc18* orp2-T4A* and *nmt-cdc18* orp2*$^+$ strains were isolated by tetrad analysis. Cdc18* was induced, and rereplication was quantitated by flow cytometry (fluorescence-activated cell sorter analysis). The *nmt* promoter is repressed for at least 10 h after removal of thiamine and is not fully induced until 16 h after removal of thiamine (49). Western blot analysis showed that Cdc18* protein was detectable 13 h after induction in both genotypes, and expression was the same in both genotypes (Fig. 5C). Data shown in Fig. 5A and B are representative of quantitative analysis of four independent isolates of each genotype. An increase in rereplication in *orp2-T4A* compared with *orp2*$^+$ was first observed when the inducible Cdc18 was wild type for Cdc2 phosphorylation sites (data not shown). However, the difference between *orp2-T4A* and *orp2*$^+$ was magnified when Cdc2 sites in Cdc18 were mutated.

Overexpression of Cdc18 is still required to drive rereplication even when Cdc2 phosphorylation sites in both Cdc18 and Orp2 are mutated as in the *nmt-cdc18* orp2-T4A* strain. One explanation for this could be that *nmt-cdc18** is not sufficient to supply replication initiation function when the *nmt* promoter is repressed; note that these cells also have a wild-type *cdc18*$^+$ gene and so are not dependent on *nmt-cdc18** for viability. We confirmed the published results that repressed levels of *cdc18** can complement the essential replication initiation function of Cdc18 in the *cdc18-K9* mutant at restrictive temperature. This suggests that that Orp2 phosphorylation and Cdc18 phosphorylation are redundant with yet more mechanisms for preventing DNA rereplication and that high level expression of Cdc18 is needed to override these mechanisms.

DISCUSSION

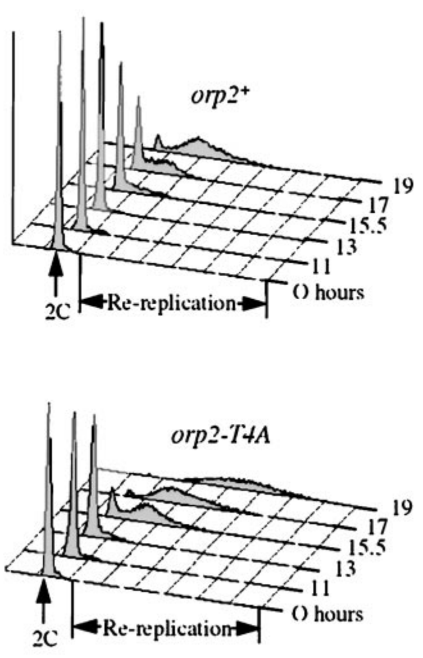
Cdc2 phosphorylation sites are present in the amino termini of Orc2 proteins from yeasts to humans. We have shown that the *S. pombe* Orc2, Orp2, is a substrate for Cdc2 in vitro and appears to be phosphorylated by Cdc2 in vivo. Mutation of Cdc2 phosphorylation sites in Orp2 results in a phenotype of enhanced rereplication when other controls preventing DNA rereplication are inactivated. We propose that phosphorylation of Orp2 is redundant with phosphorylation of Cdc18 to maintain once-per-cell-cycle control of DNA replication. Finally, our data suggest that Cdc2 phosphorylation of Orp2 is dispensable for Cdc2 activation of DNA replication.

There is evidence for phosphorylation of Orc2 proteins from budding yeast, fission yeast, and *Xenopus* to humans. As predicted from the Orp2 sequence, Orp2 can be phosphorylated in vitro by Cdc2. This Orp2 phosphorylation depends on putative Cdc2 sites. Lygerou and Nurse have shown that Orp2 is also phosphorylated in vivo, producing a change in electrophoretic mobility (45). We find that this in vivo phosphorylation likewise depends on the Cdc2 sites. The in vivo phosphorylation state of Orp2 varies according to cell cycle position, with maximal phosphorylation in the M phase and minimal phosphorylation in G_1. This corresponds to the pattern of Cdc2 activation through the cell cycle. These observations suggest that Orp2 may be an in vivo substrate of Cdc2.

The failure to phosphorylate Orp2 in the *cdc13* cyclin deletion mutant suggests that a Cdc2-Cdc13 complex is important to phosphorylate Orp2 in vivo. Failure to phosphorylate Orp2 in cells lacking Cdc13 is not easily explained as a failure of these cells to enter mitosis because other blocks to mitosis did not block Orp2 phosphorylation. That loss of the one cyclin Cdc13 nearly abolishes Orp2 phosphorylation suggests that other cyclins, such as the S-phase cyclin Cig2, are poor effectors of Orp2 phosphorylation (24, 51). Phosphorylation of Orp2 by a CDK complex primarily active in G_2/M rather than an S-phase-specific complex is consistent with a model in which Orp2 phosphorylation helps prevent rereplication during G_2/M rather than having a primary role in activating replication initiation in the S phase.

Orp2 phosphorylation was reduced but not abolished in Cdc2 mutants arrested at restrictive temperature. There are three likely causes for Orp2 remaining phosphorylated when

A.



B.

*nmt1-GST-cdc18** induction (hours)	% Re-replication	
	orp2$^+$	*orp2-T4A*
0	< 1	< 1
11	< 1	< 1
13	< 1	3
15.5	12	55
17	27	80
19	76	96

C.

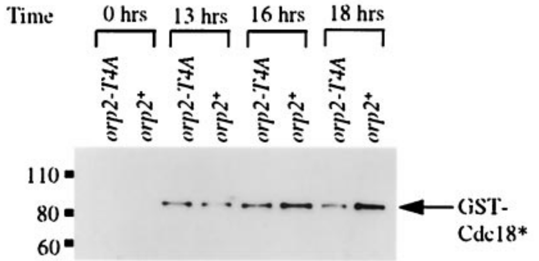


FIG. 5. Sensitivity of *orp2-T4A* mutants to rereplication. Haploid isogenic strains JLP524 *orp2*$^+$ and JLP519 *orp2-T4A* were compared upon induction of GST-Cdc18* from the *nmt1* promoter. (A) DNA content of cells after removal of thiamine to induce the *nmt1*-driven *GST-cdc18**. 2C DNA content is indicated. "Re-replication" marks DNA content greater than approximately 4C. (Top) *orp2*$^+$ GST-Cdc18* (JLP524); (bottom) *orp2-T4A* GST-Cdc18* (JLP519). (B) Quantitation of data shown in panel A. The percentage of cells with DNA content in the region marked "Re-replication" is tabulated. (C) Immunoblot analysis of GST-Cdc18* induction in *orp2*$^+$ and *orp2-T4A* mutant strains. Ten micrograms of total protein was loaded per lane. GST-Cdc18* was detected using anti-GST antibodies.

Cdc2 is inactivated. (i) There may be a kinase other than Cdc2 that carries out the phosphorylation. This is possible but seems unlikely given the importance of the Cdc13 cyclin for Orp2 phosphorylation. (ii) The mutant Cdc2 may retain some residual activity. Indeed, these temperature-sensitive *cdc2* alleles almost certainly do have residual function at restrictive temperature because complete inactivation of Cdc2 in G_2 followed by reactivation of Cdc2 leads to rereplication and diploidization, which is not seen in these *cdc2* mutants (27). (iii) Orp2 phosphorylation may be particularly stable. For instance, Cdc2 substrates might be rapidly dephosphorylated at the end of mitosis by a phosphatase analogous to Cdc14 of budding yeast. In this case, phosphates on Cdc2 substrates such as Orp2 might be quite stable until the end of mitosis. Notably, budding yeast *orc2-1* is synthetically lethal with *cdc14-1,* which could be explained if Cdc14 removes inhibitory phosphates from Orc2 (43).

Since both Cdc2 and Orp2 are essential for replication initiation, we looked for defects in the initiation of replication in the nonphosphorylatable *orp2-T4A* mutant, but no defects were seen; growth rate was normal; S phase was normal as assayed by flow cytometry; cell size at division was normal; there was no genetic interaction with the *cdc18-K9* mutant, and the transformation frequency of *orp2-T4A* mutants was, if anything, higher than that of the wild type. Finally, deletion of the first 127 amino acids of Orp2 did not obviously affect growth, suggesting that the N-terminal domain of Orp2 is not needed for initiation. Since each chromosome may have hundreds of origins of replication, even a severe reduction in the efficiency of initiation in these mutants (*orp2-T4A* and *GST-Orp2-*Δ*127*) would still allow many origins to fire, perhaps leading to normal growth rate, DNA content, and cell division kinetics. However, it is harder to explain how transformation frequency could be normal if the *orp2-T4A* mutant suffered from a significant initiation defect.

Indeed the *orp2-T4A* mutant functioned up to threefold better than wild-type *orp2* in transformation efficiency. This apparent gain of function could be explained if the Orp2 phosphorylation can inhibit initiation at least partially, especially at functional origins. For example, although Orp2 is not phosphorylated when the S phase begins, the presence of phosphorylated Orp2 at the end of mitosis might have an effect on the following S phase by delaying establishment of prereplicative complexes. The Orp2-T4A protein would suffer no such interference and so might have a higher efficiency of firing individual origins and a resulting higher transformation efficiency.

It is possible that phosphorylation of the N-terminal domain of Orp2 is important for initiation, but is completely redundant with phosphorylation of some other replication proteins. It is well known that other replication proteins (e.g., Cdc18, Mcm4, and Cdc23 [also known as Mcm10]) have clusters of Cdc2 phosphorylation sites. Thus, an initiation defect might appear only when multiple phosphorylation sites on multiple proteins are mutated simultaneously. The *orp2* mutants used in this study may be useful to investigate this possibility in the future.

In this study, we find evidence suggesting that Orp2 phosphorylation is redundant with phosphorylation of other replication proteins to prevent reinitiation of replication. We were more successful in finding a phenotype when we looked for rereplication because we had some knowledge of other pathways that prevent rereplication and could deregulate those pathways. This deregulation was achieved by overexpression of Cdc18* from the *nmt* promoter, a condition in which both the transcription and phosphorylation of Cdc18 are deregulated and cells are driven to rereplicate DNA. Both the kinetics and magnitude of rereplication were affected by mutation of Cdc2 phosphorylation sites in Orp2. Rereplication occurred 4 to 6 h after the appearance of Cdc18* in the wild-type strains but only 2 h after the appearance of Cdc18* in the *orp2-T4A* strains. Thus, the *orp2-T4A* mutant is sensitized to rereplication, showing that the phosphorylation sites in Orp2 help to prevent rereplication.

The idea that phosphorylation of Orp2 is more important for inhibiting reinitiation of replication than for triggering initiation is consistent with the timing of Orp2 phosphorylation and with its dependence on *cdc13*$^+$. Orp2 phosphorylation is first detected at about the time of the S phase, but is maximal in G_2 and M when initiation is no longer occurring and when reinitiation must be prevented. Orp2 phosphorylation is not seen in the *cdc13* mutant; this mutant replicates (suggesting phosphorylation of Orp2 is not essential for replication) and rereplicates (consistent with the idea that phosphorylation of Orp2 helps prevent rereplication).

The idea that phosphorylation of Orp2 helps prevent reinitiation is also consistent with Orc2 phosphorylation and ORC-CDK interactions in other systems. In *Xenopus* extracts, XOrc2 is unphosphorylated during interphase and becomes highly phosphorylated during mitosis (9). *Xenopus* ORC fails to bind the Cdk2-cyclin A or Cdk2-cyclin E complexes known to be important for activating initiation of replication but does bind the Cdc2-cyclin A complexes that might be important for preventing reinitiation (65). Finally, mutation of the consensus CDK phosphorylation sites in budding yeast Orc2 together with mutation of sites in Orc6 also sensitizes cells to rereplication (J. J. Li, personal communication).

In current models for replication initiation, the Orc proteins form a seemingly static platform onto which other initiation factors are assembled. How then might Orp2 phosphorylation inhibit reinitiation of DNA replication? Since binding of the ORC to DNA is not regulated and Orp2 is not targeted for destruction (34, 36, 45, 57), phosphorylation of Orp2 probably alters binding to other initiation factors (e.g., Cdc18) or promotes changes within the ORC complex. Our favored model is that the N-terminal domain of Orp2 is activated by phosphorylation to inhibit initiation, perhaps by binding a negative regulator analogous to geminin, or by interfering with the binding of other initiator proteins to the ORC (50, 69, 73). Another model is that the N-terminal domain of Orp2 has a positive function that is then inactivated by phosphorylation. This second model is hard to reconcile with the fact that most of the N-terminal domain of Orp2, including the phosphorylation sites, can be deleted without abolishing Orp2 function. One prediction of either model is that phosphorylated Orp2 would not initiate DNA replication. However, the *orp2-T3D* allele created to try to mimic Orp2 phosphorylation shows only a very weak initiation defect. Our simple models may be incorrect or the *orp2-T3D* mutant may fail to mimic the phosphorylated state of Orp2. Identifying what factors interact with the N terminus of Orp2 would help clarify exactly how phosphorylation modulates the ORC function.

In *Xenopus* and humans, the homologue of Cdc18, Cdc6, is not fully degraded during the S phase (reviewed in reference70). In these organisms, mechanisms for preventing reinitiation that are independent of Cdc6, such as phosphorylation of Orc2, may be even more important than they are in yeasts. Since CDK phosphorylation sites in the amino terminus of Orp2 are a conserved feature of Orc2 and its homologs, the phosphorylation of Orc2 may be a conserved mechanism for maintaining once-per-cell-cycle control of DNA replication. Of course, there must also be additional controls (since, for instance, the *orp2-T4A* mutant does not rereplicate unless Cdc18* is overexpressed). Multiple overlapping controls provide extra assurance that cells will not reinitiate replication. Any one control may be sufficient to prevent bulk rereplication of DNA but might allow occasional rereplication events from some origins. Such rare events would not be readily detectable in the laboratory except by specially designed genetic tests, but the genomic instability caused by even very rare reinitiation might reduce fitness in simple eukaryotes such as yeast and possibly lead to genetic diseases and cancer in humans.

ACKNOWLEDGMENTS

We thank Paul Russell and Antonia Lopez-Girona for intellectual inspiration and reagents. Thanks go to Beth Baber, Clare McGowan, and Kazuhiro Shiozaki for friendship and helpful advice critical to the success of the in vitro phosphorylation experiments. Thanks go to Joan Kiely and Anya Bogomez, whose experiments contributed to this work. Thanks go to Joachim Li for communicating results prior to publication. Special thanks go to Bruce Futcher for extensive help with the manuscript and to Nancy Reich for helpful comments.

This work was supported by a Beckman Scholars Award to W.M., Leukemia Society Special Fellow grant and Kimmel Scholar Award to J.L., and NIH grant R01 GM61532.

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